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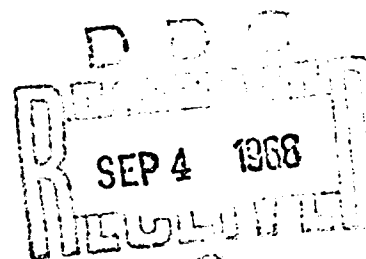
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From the Hygienic-Bacteriological Institute of the University of Bern
(Prof. Dr. C. Hallauer, Director)

DETERMINATION OF YELLOW-FEVER VIRUS-HEMAGGLUTININ IN
HUMAN EXPLANTS

by

C. Hallauer and G. Kronauer

With six illustrations (received on 15 April 1960)

The breeding of yellow-fever virus in human tissue explants has been reported on earlier (13, 15). In order to determine the existence of a virus it is generally sufficient to determine the cytopathogenic effect whose specificity if necessary can be ascertained through the inhibition test using anti-sera.

(The cytopathogenic effect can easily be determined in rolled culture tubes, primarily in the peripheral zone of the outgrowing tissue. Streak-shaped bands of tissue defects (in the rolling direction of the little tube) can be determined macroscopically rather early and can be considered as unfailing signs of virus infection.)

The titration of the infectiousness of the culture virus gives us roughly the same values in the explant and in the cerebral mouse test. 17-D strains constantly passed in the explant however lose their mouse pathogenicity so that we can no longer make a comparative infectiousness test in the explant and in the animal experiment.

It would therefore be desirable to establish hemagglutinin in the virus-infected explant and this certainly did not appear hopeless particularly in view of the considerable virus content which was achieved in vitro. After a few initially unsuccessful preliminary experiments we were able to achieve this goal, that is to say, we were able to work out a very simple method according to which virus-specific hemagglutinin can be established in the infected tissue and, most recently, also in the cell-free phase of the culture medium.

Material and Method

Virus strains. The following culture passage strains, some of which had been described earlier, were used in these experiments:

17 D (London) of the 99th HoLa "Slp" /50th-60th KB passage,

17 D (Amsterdam) of the 52nd-60th KB passage,

Asibi I of the 49th-65th KB passage, respectively 47th KB/1st - 15th Amnion FL passage,

Asibi II of the 8th-21st KB passage.

The 17-D London, respectively, Amsterdam strains did not reveal any mouse pathogenicity any longer. The Asibi I strain had apparently lost its viscerotropia for monkeys through sustained passage in the explant. The strain Asibi II represents a second culture passage strain of the same initial virus (lyophilized monkey serum, Rockefeller Institute, New York) and was refreshed prior to transfer to the explants by means of an additional monkey passage. The monkey pathogenicity of this strain had not yet been determined.

Explants. All virus strains were passed in KB explants which had earlier proved to be optimum for the breeding of yellow-fever virus. Occasionally and for the purpose of comparison we also used HeLa "Zürich" explants for virus passage. Finally we also tested the Amnion FL strain of Fogh (12) for its suitability in virus breeding.

The culture medium was always the same for the virus-infected explants and had the following composition:

Calf serum	10.0	Penicillin 500 E/cc
Lactalbumin 5%	10.0	Streptomycin 50 g /cc
Medium 199	10.0	Nystatin 100 E/cc
Hanks Solution	70.0	pH 7.4 - 7.6

The same medium was also used for the transfer and maintenance of KB and HeLa "Zürich" cell strains while the strain cultures of the Amnion FL tissue were passed in the Ly medium mentioned by Fogh (12).

The strain cultures were incubated at a temperature of 37° C in steadily held bottles (13 cc of culture medium), while the experimental cultures were kept in little tubes (2.5 cc culture medium) in the rolling apparatus.

The virus infection of the explants as well as the evaluation of the cytopathogenic effect were performed in the manner described earlier (see also preceding reference to determination of cytopathogenic effect).

Determination of Hemagglutinin

1. In Tissue

(a) Borate extraction. The tissue explanted in the little rolling tube is washed once -- after removal of the culture medium -- with 2.5 cc, each, of Hanks solution (pH 7.4 - 7.6) and is then covered, in situ (diagonally positioned culture tube) with 2.5 cc, each, borate-NaCl solution (0.05 M borate - 0.12 M NaCl, pH 8.7 - 9.0) (the buffer solution, by the way, is prepared according to the indications by Clarke and Casals (11)); it is then extracted for 30 minutes at room temperature (20° C). After the removal of

the hemagglutinin-containing borate extract, the explants are washed once with Hanks solution (pH 6.5) and they are then provided with a new culture medium.

The borate extraction can also be performed in the centrifuged cell sediment of processed cultures. Prior washing of the cell sediment with Hanks solution is required only if the superposed medium cannot be completely transferred with a pipet or if the tissue was homogenized with trypsin.

(b) Phosphate extraction. Instead of borate we use a phosphate, respectively, phosphate-borate buffer (0.2 M phosphate - 0.15 M NaCl; 0.05 M borate - 0.12 M NaCl, pH 6.6 - 6.7) for the extraction of the hemagglutinin. Prior to the extraction, the tissues are once again washed, just once, in situ, using Hanks solution (pH 6.6); after that they are extracted with 2.5 cc, each, buffer solution for 20 minutes at room temperature; we then wash once with Hanks solution (pH 7.4 - 7.6) and we finally supply 2.5 cc, each, of new culture medium.

A modification of this procedure consists in the following: we leave the previously washed tissue in situ in contact with 2.5 cc, each, of phosphate buffer (pH 6.6 - 6.7) plus goose erythrocytes (1%) during 20 minutes at room temperature. The erythrocyte suspension is then removed from the tissue as completely as possible (using an injection syringe and it is then poured into sedimentation, respectively, critical centrifugation tubes. The explants are then once again washed with Hanks solution (pH 7.4 - 7.6) and are provided with new culture medium.

2. In the Medium, After Prior Treatment with Freon

The culture medium to be tested is brought to a pH of 7.4 - 7.6 by means of NaHCO₃; the cell elements are removed by means of centrifuging and it is then cooled to 4° C in an ice-cooled water bath. After adding the same volume of ice-cooled freon solution (1,2 difluorotetrachlorethane) the mixture is forcefully agitated for 1-2 minutes in the cold environment and is then centrifuged for 2 - 3 minutes at 2,500 r/min. The watery supernatant liquid is then once again shaken once and perhaps twice with freon. This method makes it possible to remove nonspecific, hemagglutinin-inhibiting substances.

Conservation of hemagglutinin extracts. Borate extracts with a pH of 8.7 - 9.0 can be conserved at -20° C. This applies also to media agitated with freon. On the other hand, phosphate extracts with a pH of 6.6 - 6.7 cannot be preserved and must therefore be used immediately after they are obtained.

Hemagglutination test. The hemagglutinin extracts are diluted in the customary little test tubes in 0.25 cc, each, of phosphate-borate buffer with a pH of 6.7; the dilution here is 1:2 all the way to 1:1024; they are then transferred (mixed) in the same buffer with 0.25 cc, each, of a 0.75% goose erythrocyte suspension (Porter-Mold, 10). After the mixtures have

stood at room temperature for 1/2 - 1 hour, the hemagglutinin titer is determined.

Hemagglutination inhibition test. Hyper-antisera of monkeys and rabbits (we want to thank Mr. H. Theiler, New York and Mr. I.S. Porterfield, London, for letting us have these sera) were pre-treated with acid-washed kaolin according to the method described by Clarke and Casals (11) for the removal of nonspecific inhibitors. They were then diluted with borate buffer (pH 8.7 - 9.0) 1:10 all the way to 1: 5120. We took 0.25 cc, each, of these serum dilutions and mixed them with 0.25 cc, each, of borate extract, respectively, 8 hemagglutinin units; the mixtures were left standing for 4 hours at +4° C. After that we added 0.5 cc, each, of a 1% goose erythrocyte suspension in phosphate-borate buffer (pH 6.7) and we read off the inhibition titer after the mixture had stood at room temperature for 1/2 hour.

Experiments

1. Obtaining Hemagglutinin

The original intention of establishing hemagglutinin in the virus-infected tissue with illegible passage in photostat proved impractical because virus-containing explants could certainly not adsorb goose erythrocytes which had been added and which were suspended in a phosphate buffer solution with a pH of 6.7. In these preliminary experiments we made the surprising discovery that erythrocyte suspensions of this kind hemagglutinated very intensively within a very short period of time after having been kept in contact for 15 - 20 minutes with virus-containing tissue in vitro. The very obvious assumption -- to the effect that the explanted tissue releases virus-specific hemagglutinin under the effect of the acid phosphate buffer -- could be confirmed through the successful hemagglutinin extraction with phosphate buffer solution. The fact that the release of the hemagglutinin is not brought about by the acidity of the buffer emerges from the observation according to which a hemagglutinin extraction with Hanks solution of corresponding acid reaction (pH 6.7) is not possible. The effect observed would, instead, seem to be brought about by the cytotoxic effect of the phosphate solution; at any rate, explants reveal a morphologically changed appearance after phosphate extraction (temporary rounding of tissue cells).

The phosphate extraction of yellow-fever hemagglutinin involves two disadvantages: first of all, the previously mentioned tissue damage which in case of repeated extraction attempts can lead to the loss of the explant and, second, the great instability of the hemagglutinin in the acid extract, which requires rapid utilization. For the routine hemagglutinin determination we therefore extensively refrained from using the phosphate buffer extraction, that is to say, the extraction was confined to those cases in which a rapid, qualitative hemagglutinin determination was desired. For this, the advance treatment of virus-infected explants with goose erythrocytes, suspended in a phosphate buffer solution, is highly suitable as the most sensitive test.

The method of selection was finally the extraction in the alkali range, which had been tested by Sabin and associates (5 - 9) in obtaining hemagglutinin in other arbor viruses, using a borate buffer with a pH of 8.7 - 9.0. This extraction method offers a number of major advantages, specifically, the surprisingly small cytotoxic effect of this buffer, the apparently quantitative release of hemagglutinin from the virus-infected tissue and the considerable stability of the hemagglutinating extracts. The fact that in this case the alkalinity of the buffer is decisive for the extraction effect is indicated by the following: a Hanks solution with a corresponding pH is likewise suitable as extraction means, although apparently to a lesser degree.

The establishment of hemagglutinin in the liquid culture phase, respectively, in the culture medium created major difficulties in the beginning. Repeated experiments, aimed at removing the nonspecific inhibitors of the medium -- whose hemagglutination inhibition titer should be suggested at 1:64 -- by means of Seitz filtration (4) did not lead to any clear results. Finally the repeated shaking of the virus-containing culture liquid with fluorocarbon, respectively, freon, in a cold environment proved to be a method suited for the isolation of hemagglutinin. Borate extracts can likewise be freed of any still existing inhibitors through prior treatment with freon, without any adverse effect on the hemagglutination titer.

2. Properties of hemagglutinin.

(a) Behavior During Hemagglutination Test

The activity of hemagglutinin -- as in all arbor viruses -- depends to a high extent on the hydrogen ion concentration in the reaction environment. The optimum pH-value is definitely around 6.6 - 6.7; at a pH of > 7.0 and < 6.4 we mostly do not have any hemagglutination. The effect of temperature on the process of hemagglutination was investigated at $+4^{\circ}$, $+20^{\circ}$, and $+37^{\circ}\text{C}$; at room temperature ($+20^{\circ}\text{C}$) and in cold weather ($+4^{\circ}\text{C}$) we achieved the same hemagglutination titers and the temperature effect was confined to the different speed of the reaction; at 37°C (in the water bath), on the other hand, there is no hemagglutination at all, provided the reaction components were pre-heated to this temperature before mixing for a short time (5 minutes). Erythrocytes of geese -- in accordance with the statements by Porterfield (18) -- are ideal for the establishment of yellow-fever hemagglutinin; chicken erythrocytes (taken from adult animals) are agglutinated only irregularly and, at best, in low titers. The concentration of the erythrocyte suspension -- as was likewise established for the other arbor viruses (6, 19) -- is decisive for the sensitivity of the test; a reduction of the erythrocyte density down to one half ($1.0 - 0.5 - 0.25$) is paralleled by a corresponding increase in the titer by one (1:2) dilution stage. At room temperature the hemagglutination develops rather quickly; in the higher hemagglutinin concentrations, the agglutinates can be determined with the magnifying glass after as little as 10 minutes and we can read off the titer limit after another 20 minutes. The hemagglutinates thus formed mostly come in the form of a more or less dense wall lining which -- in strongly positive specimens -- can come off in the form of lamella, coupled with the

formation of a bottom sediment. No indications for an enzymatic reduction of cell receptors could be found. Agglutinates agitated several times can, to be sure, be transferred to a homogeneous, spontaneously not agglutinating cell suspension which, however, is re-agglutinated in its original strength through the renewed addition of fresh hemagglutinin. The virus specificity of the hemagglutinin was ascertained in the inhibition test, using inhibitor-free yellow-fever antisera; here we determined an inhibition titer of 1:320 (rapid serum), respectively, 1:1280 (monkey hyper-antiserum).

(b) Behavior in Explant

If we want to establish hemagglutinin, it is very important to know whether the infected explants are held stable or whether they are moved in the agitator; we can determine the hemagglutinin regularly only in the latter; in the former case this is impossible most of the time. The obvious assumption -- according to which we could achieve a higher infectiousness titer in agitated cultures than in resting cultures -- could be verified by means of a comparative infectiousness test involving identical infected explants (100 tissue culture doses of virus Asiatic I); the titer difference, which was determined on the 5th day p.i. was about log 2.0 (6.5:4.3) for the tissue (borate extract) and it was roughly log 1.0 (5.6:4.5) for the culture medium (native). In stably incubated explants we therefore obviously do not achieve the virus concentration (see below) which is required to determine the hemagglutinin -- at least not as a rule. All experiments were therefore performed in agitated explants.

Hemagglutinin formation time. In order to determine the time at which we might be able to establish the presence of hemagglutinin for the first time and in order to determine the maximum titer achieved as well as the duration of the hemagglutinin production process, we continuously, that is to say, daily, subjected the explants, which had been infected with varying doses of viruses (log 0.0 to 6.0 ID₅₀), to borate buffer extraction. The experimental results achieved here are shown in Table 1 and in the Graphs 1 - 6 (see Appendix).

As expected, the volume of the inoculation dose determines the incubation time at which we can make our first hemagglutinin determination. In explants, which were inoculated with larger infection doses (10⁶ to 10³ ID₅₀), this time usually comes on the third day and in a few rare cases on the second day, p.i.; (in case of very high inoculation doses, the hemagglutination formation is very clearly inhibited and on occasion it is completely suppressed; Chanock and Sabin (8) made a similar discovery in the case of the virus of equine encephalitis, type West; no hemagglutinin could be extracted from massively inoculated mouse brains); less strongly infected cultures can give us a hemagglutination determination on the 4th day, p.i., at the earliest. In each case we can establish the hemagglutinin in the explanted tissue 24 - 48 hours before the first indication of a cytopathogenic effect. The maximum hemagglutinin titer is achieved in most cases within 24 hours after the first possible determination and as far as time is concerned coincides with a medium-level cytopathogenic effect (++++ reaction: increasing rounding of tissue

cells, clouding of medium). The duration of the hemagglutinin production depends on the type of explanted tissue, that is to say, on its survival capability. KB explants are completely destroyed within a short period of time (1 - 2 days) after the maximum virus titer has been achieved so that the hemagglutinin content of the tissue rapidly drops to a minimum. In HeLa-"Zürich" and Amnion "FL" explants, on the other hand, the cytopathogenic effect revealed a pronounced protracted course and as a rule did not end in the complete destruction of the tissue (*). An increase and a drop in the hemagglutinin concentration take place also in such explants at a correspondingly slower rate.

TABLE 1

TIME DURATION OF HEMAGGLUTININ FORMATION IN EXPLANTED TISSUE
AS A FUNCTION OF THE INOCULATED DOSE

(f)	Impfdosis (TCD)	(a) (d) (e)	Haemagglutinin		(b) Termin des ersten Nachweises*						(c) Termin des maximalen Titers*							
			Virusstämme		17 D		Asibi I		Asibi II		17 D		Asibi I		Asibi II			
			Gewebe		KB.		KB		Amnion		KB		HeLa		KB		HeLa	
			1	5.6	5.0	6.4	5.1	6.3	5.7	5.4	7.0	5.5	7.2				
			10	4.6	4.2	4.7	4.1	5.3	5.5	5.0	6.6	5.5	6.2				
			100	4.4	3.4	4.1	3.6	5.2	4.7	4.7	5.2	4.1	5.8				
			1,000	3.0	3.0	3.7	3.5	4.5	4.2	4.0	4.8	4.0	5.0				
			10,000	3.0	3.0	3.0	3.0	3.5	4.0		5.0	3.0	4.5				
			100,000	2.7		3.0		3.0	3.0		3.5		3.5				
			1 Mill.		2.0		3.0			3.0		3.0					

Legend: a-- hemagglutinin; b-- time of first determination (*); c-- time of maximum titer (*); d-- virus strains; e-- tissues; f-- inoculation dose (TCD); (*) number of days after infection (average values).

(The further breeding of surviving HeLa and Amnion cells mostly does not create any difficulties and enables us to obtain "permanent cultures" (several months old, not converted or transferred explants). Explants of this kind are in a state of equilibrium between proliferative tissue growth and degenerative cell decomposition and are chronically infected, as we were able to see on the basis of repeatedly successful virus and hemagglutinin determinations as well as on the basis of the proven resistance against re-infection. An extensively similar finding was made in connection with the breeding of classical and atypical poultry pest virus [chicken cholera] in explants of the same type (16, 17, 14).)

The virus strains used do not essentially differ from each other with respect to their hemagglutinating capability although the particular maximum titer achieved in each case in the Asibi strains was on the average somewhat higher than in the case of the 17 - D strain. We might mention here merely

that the 17 - D strain (London), which had been passed longest in human explants, no longer produced any hemagglutinin after 99 HeLa - "Sip" and 54 KB passages. A similar loss of hemagglutinating activity was also reported by Sabin et al. (19, 20, 8) in old passage strains of other Arbo viruses. In the case here it is now highly questionable whether the culture strain actually lost its capability for hemagglutinin formation because there was a simultaneous drop in the infectiousness titer by about 1 log₁₀ so that, possibly, only the threshold value required for hemagglutinin determination was not reached.

(The fact that this assumption would seem to apply here emerged during the 69th KB passage in which hemagglutinin -- corresponding to a new rise in the infectiousness titer -- once again could be established in a very small quantity.)

The finding that the hemagglutinin determination can be made also in the culture medium -- following prior elimination of nonspecific inhibitors through freon -- offered a possibility for making a simultaneous and continuous determination of the quantitative distribution of hemagglutinin over the tissue and the liquid culture phase. Hemagglutinin extraction from the infected tissue, as in the earlier experiments, involved the superposition of the washed explant with 2.5 cc, each, of borate buffer with a pH of 8.7 over 30 minutes at room temperature; to establish the hemagglutinin in the medium, 2.5 cc, each, of cell-free culture liquid were agitated with freon in a cold environment (+ 4° C) three times in succession. The efficiency of this method can be seen in Table 2. Partially cleaned specimens as a rule reveal a lower inhibition zone which occasionally can also be found in the uncleaned, virus-containing culture media. The purification or cleaning process can be considered completed when we can no longer establish the existence of a prozone and when the hemagglutinin titer remains at a constant level.

TABLE 2

PURIFICATION OF CULTURE MEDIA WITH FREON
Hemagglutinin titer before and after 1 - 3-fold Freon agitation

(a)	Explant No.		5769				5769/64				5769/70				6906			
	Gewebe		HeLa				HeLa				HeLa				K11			
	Virusstamm		Asibi II				Asibi II				Asibi II				Asibi II			
	Freonbehandlung		(+)	1x	2x	3x	Vor	1x	2x	3x	Vor	1x	2x	3x	Vor	1x	2x	3x
(c)	Hemagglutinations- Test Verdünnungen	1: 1	-	(+)	++	++	-	-	(+)	++	-	-	(±)	++	-	(±)	++	++
		1: 2	-	++	++	++	-	-	+	++	-	-	(+)	++	-	++	++	++
		1: 4	-	++	++	++	-	(+)	++	++	-	-	+	++	-	++	++	++
		1: 8	-	++	++	++	-	+	++	++	-	++	++	++	-	++	++	++
		1: 16	-	++	++	++	-	++	++	++	-	++	++	++	-	+	++	++
		1: 32	(+)	++	++	++	+	+	++	++	-	+	(+)	+	-	(-)	(+)	++
		1: 64	(+)	++	++	++	(±)	(+)	+	+	-	(+)	(+)	(+)	-	-	(±)	++
		1: 128	(+)	++	++	++	-	-	(±)	(±)	-	-	-	-	-	-	-	++
		1: 512	-	+	(+)	(±)	-	-	-	-	-	-	-	-	-	-	-	(+)
		1: 1024	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Legend: a-- explant number.; b-- tissue ; c-- virus strain; d-- freeze treat-
ment; e-- hemagglutination test dilution; f-- before; evaluation: +++ heavy
marginal coating with lamellar separation; ++ dense marginal coating; + fine
marginal coating; (+) weak marginal coating; (±) slightly indicated marginal
coating.

TABLE 3

HEMAGGLUTININ CONTENT OF TISSUES (TORNATE EXTRACT) AND MEDIUM
(TRENON PURIFICATE) IN KB-, HeLa- AND AMBION EXPLANTS. VIRUS
STRAINS: ASBIB I AND II

(a) Explant		(b) Growth		Virus	(c) Inoculum (TCID ₅₀)	(d) Inc. p.i.	CFZ	(e) Hemagglutinin	
No.	Passage	No.	Passage					(f) Tissue	(g) Medium
KB	6055/36	57.	57.	Asibi I	10	4. 5. 6.	++ ++ ++	1.2 2.6 2.4	0.8 1.5 1.8
	8101/92	57.	57.	Asibi I	100	3. 4. 5. 6.	++ ++ ++ ++	0.9 2.7 2.7 1.8	0.0 1.5 1.8 1.7
	8107/98	24.	24.	Asibi II	10	4. 5. 6.	- ++ ++	0.5 2.4 1.8	0.0 1.5 1.5
	6113/14	24.	24.	Asibi II	100	4. 5. 6.	- ++ ++	1.2 2.4 1.5	0.9 1.5 2.1
	5781/62	20/2.	20/2.	Asibi II	100	5.	++	2.1*	1.8
	5767/63	20/2.	20/2.	Asibi II	100	5.	++	1.8*	1.8
	5763/64	20/2.	20/2.	Asibi II	10	6.	++	2.1*	2.1
	5769/70	20/2.	20/2.	Asibi II	10	6.	++	1.8*	1.2
HeLa "Zürich"	5955	20/3.	20/3.	Asibi II	10	5. 6.	++ ++	2.1 1.2	1.2 2.1
	5963	20/3.	20/3.	Asibi II	10	6. 7.	++ ++	1.8 1.2	1.2 1.8

HeLa "Zürich"	6070/71	20/4.	Asibi II	100	2. 3. 4. 5.	- + ++ +++	0.4 0.0 1.2 2.1 1.8 1.5
Amnion "FL"	5933	47/17.	Asibi I	10,000	5. 6. 7. 8.	(+) ++ +++ R	3.0 2.1 1.2 -
	5934	47/17.	Asibi I	1,000	5. 6. 7. 8.	(+) ++ +++ R	3.0 2.1 0.9 -
	5935	47/17.	Asibi I	100	5. 6. 7. 8.	- + +++ R	1.5 2.7 2.7 0.9
	5936	47/17.	Asibi I	10	5. 6. 7. 8.	- + +++ R	0.9 - 2.7 0.9
	5937	47/17.	Asibi I	1	5. 6. 7. 8. 9. 10.	(+) ++ +++ R	- 2.4 2.7 1.8 0.9 -

Legend: a-- explants; b-- tissue; c-- inoculation dose (TCD); d-- day, p.i.; e-- hemagglutinin titer; CPE-- cytopathogenic effect; R-- purification of explant, surviving tissue; - : cannot be determined; * -- maximum titer achieved.

A comparative investigation on the hemagglutinin concentration in explanted tissue, on the one hand, and the pertinent culture medium, on the other hand, starts from the assumption that the borate extraction approximately exhausts the entire hemagglutinin content of the tissues and that the freon treatment of the medium likewise renders the hemagglutinin present in each case quantitatively determinable. There are two findings which speak in favor of this assumption: (1) the tissue extracted with borate buffer mostly releases only traces of hemagglutinin in connection with a subsequent second extraction (as we were able to find out later on the one-time borate extraction of a tissue suspension is not always exhaustive because a repeated extraction occasionally is capable of releasing considerable quantities of hemagglutinin in case the cell suspension is frozen at -20°C in the borate buffer for 24 hours and then quickly thawed out) and (2) the borate extracts with high hemagglutinin content reveal infectiousness titers which can be considered maximum for the yellow-fever virus. The same applies to freon-purified substances (freon purificates) for which the same infectiousness titers as for the corresponding non-purified initial media are determined as a rule.

Of the numerous comparative titrations of this kind we have reproduced the results in a limited number in Table 3.

As was to be expected a priori, the hemagglutinin of the culture medium -- in comparison to the tissue medium -- reaches the threshold of determinability and titer increase about 24 hours later. The hemagglutinin concentrations proved in the medium extensively correspond to the progression of the cytopathogenic effect so that the assumption is justified that the intensity of the tissue decomposition is decisive for the extent of the virus release into the liquid culture phase. In general, we determined lower maximum titers in the culture medium than in the corresponding tissue. In Amnion explants, in which the cytopathogenic effect is considerably delayed, this finding would correspond to actual conditions; in other explants (KB, HeLa) -- particularly and primarily in those which were not subjected to a prior borate extraction -- we were however able to observe repeatedly that the same maximum titers were achieved in the tissue and in the medium. The frequently established relatively small hemagglutinin content of the culture medium is therefore possibly the consequence of the artificial virus removal through prior borate extractions of the explanted tissue.

(c) Relationship Between Hemagglutinin and Infectiousness Titer

Hemagglutinin-containing extracts from infected tissues are just as infectious for explants (and for the white mouse) as cell-free culture liquids. We therefore increasingly frequently used borate extracts for virus passages in the explant so that the hemagglutinin titer of the inoculated virus extract could be compared to the infectiousness titer which is achieved

in the explant (and occasionally also in the cerebral mouse test). Freon-purified substances [Freon purificates] likewise revealed the same infectiousness titer as the non-purified initial media so that in this case likewise the ratio of hemagglutinin to infectiousness titer can be determined. In Table 4 we have the titer values determined for borate extracts.

TABLE 4

HEMAGGLUTININ AND CORRESPONDING INFECTIOUSNESS TITERS --
BORATE EXTRACTS FROM KB EXPLANTS -- VIRUS STRAIN: ASTER I.

(a) I. Durchschnittswerte*		(b) II. Einzelversuche				
Titer (- log)		(c) Explantat Nr.	(d) Tag p.i.	CPE	Titer (- log)	
HA	ID ₅₀				HA	ID ₅₀
—	<4.5	5301-03	5.	+	1.8	6.0
0.3-0.9	4.5-5.0		6.	+++	—	7.0
1.2	5.0-5.5	5709	3.	—	—	4.0
1.5	5.5-6.0		4.	+	1.5	5.8
1.8	6.0-6.5		5.	++	2.1	7.0
2.1	6.5-7.0		6.	+++	—	6.0
2.4-3.0	>7.0					

Legend: a-- I. average values (*); b-- II. individual experiments; c-- explant number; d-- day, p.i.; HI -- hemagglutinin titer; ID₅₀ -- infectiousness titer in explant; CPE -- cytopathogenic effect; (*) -- calculated on the basis of 28 experiments; - : cannot be determined.

The average values of the titers, which were computed from a larger number of individual experiments, leave no doubt that there is a certain proportionality between the hemagglutinin concentration and the level of infectiousness; an increase in the hemagglutinin titer by 0.3 log generally is paralleled by an increase in the infectiousness by roughly 0.5 log. The threshold of determinability of hemagglutinin lies between 4.5 and 5.0 log₁₀ infection doses (ID₅₀) for the explant and is thus strikingly low. Table 4 also shows us two individual experiments in which the titer ratio was traced in terms of time; here we made the rather noteworthy finding that no hemagglutinin could be established anymore at the time of the maximum cytopathogenic effect, although the infectiousness titer continued to increase or dropped only to a comparatively small degree. It remains to be explained and clarified whether such a discrepancy between hemagglutinin and infectiousness can be proved frequently or regularly during the final stages of infected explants.

Corresponding comparative titer determinations with freon-purified culture media have so far been made only to a very small extent. In the case of five purificates tested, we determined an entirely similar ratio between

the hemagglutinin and infectiousness titers (- log 1.5:5.5; 1.5:5.6; 1.8:6.0; 1.8:6.3; 2.1:6.6) as in the case of the tissue extracts.

(d) Stability, Reactivity, Conservability

Systematic investigations on the stability of the yellow-fever hemagglutinin at various temperatures and variable pH values have been made only to a limited extent; this is particularly interesting since we already have some information on this (9, 10). Along with other Arbor viruses, the hemagglutinin of the yellow-fever virus shares the property of maximum lability in the pH range (6.6 - 6.8) of its optimum activity. Phosphate and borate-phosphate buffer extracts with a pH of 6.7 reveal a rapid drop in the hemagglutinin titer at room temperature (20° C) within 1 - 2 hours; at lower temperatures (+ 4°, - 20° C) there is likewise a complete activity loss within 24 hours. On the other hand, hemagglutinin has a relatively high degree of stability in case of alkaline reaction. Borate extracts with a pH of 8.7 - 9.0 can be conserved without any major titer loss for 1 - 2 days at + 4° C, and for 4 - 5 days at - 20° C. In case of longer storage, the hemagglutinin titer as a rule drops progressively so that the specimens finally become completely inactive -- at + 4° C mostly from the 6th - 10th day on, at - 20° C mostly from the 9th - 15th day on. The stability of the hemagglutinin by the way also fluctuates considerably from one specimen to the next so that the times given here only refer to an average norm. Borate extracts can be lyophilized without major titer loss; the duration of the conservability of lyophilized specimens however has not been determined as yet. Finally we come to the question as to whether in such investigations the stability of the hemagglutinin is in fact decisive or whether the drop in the hemagglutinin titer is brought about only through the progressive loss of infectiousness during the conservation period. On the basis of several infectiousness determination involving cold-conserved specimens we would have to assume that the "inactivation of hemagglutinin" takes place in terms of time when the infectiousness titer drops below the threshold value required for hemagglutination.

Discussion of Experimental Results

According to Sonja M. Buckley (1) certain Arbor viruses of Group A (equine Encephalomyelitis Type West, Sindbis) respectively B (Kyasanur Forest Disease, West Nile) in the infected explant of animal and human origin (chicken embryo, HeLa, Detroit-6, embryonal, human intestinal tissue) can be determined and established with the help of the hemadsorption test of Shelokov, Vogel, and Chi (21). We were never able to observe a true hemadsorption with the yellow-fever virus (that is to say, a firm binding of added goose erythrocytes to infected, explanted tissue) although we strictly followed the technique recommended by Buckley. Under the effect of the alkaline borate buffer the virus, respectively, the hemagglutinin, were always completely washed out of the infected tissue cells so that no hemadsorption phenomenon could take place precisely because of this. But there was no hemadsorption either, whenever the pretreatment of the explant with a washed borate buffer was omitted and when the infected tissue was placed in contact only with an erythrocyte suspension

prepared in a Hanks solution with a pH of 6.7. Although in this case the environmental conditions for the determination of yellow-fever hemagglutinin have been met, it was impossible to prove a hemagglutination effect either on the surface of the explanted tissue or in the liquid culture phase. On the basis of these findings it seems reasonable to assume that the hemagglutinin of yellow-fever virus is not spontaneously liberated of the still vital, morphologically intact cells and that it instead becomes determinable only after the cell decomposition or due to artificial extraction. It remains to be determined whether the yellow-fever virus differs basically from the other Arbor viruses in this respect, as one might assume on the basis of the experiments by Buckley. Buckley likewise succeeded in achieving the determination of hemagglutinin only by using a hemagglutinin-extracting borate buffer; the "hemadsorption," which she established, is undoubtedly not of the "purely classical type" because agglutinates could be established also outside the explanted tissue in chain-shaped groups.

The most important result of this investigation probably resides in the fact that we succeeded in extracting the hemagglutinin from infected explants apparently quantitatively, without the tissues here being damaged to any great extent. It is furthermore worth noting that agitation with fluorocarbon (freon) eliminates nonspecific inhibitory substances and this also makes it possible to prove the existence of the hemagglutinin present in the culture medium. We thus have two comparatively simple methods available for the early establishment of the yellow-fever virus in the explant -- even before the appearance of the cytopathogenic effect. Both methods are suitable also for the purification of hemagglutinin, respectively, virus, and could therefore prove to be valuable also for electron-optical preparation and possibly also for the obtention of albumin-poor vaccines.

The relationship of the hemagglutinin to the infectious virus particle has so far not been investigated. At any rate it is certain that borate extracts and freon purificates contain not only hemagglutinin but also infectious viruses and that there is an extensive proportionality between the hemagglutinin concentration and the degree of infectiousness.

The properties of the yellow-fever hemagglutinin, which so far have been investigated predominantly for the purpose of obtaining an antigen suitable for the hemagglutination inhibition test (3, 4, 10) basically reveal the criteria set up by Sabin and associates for other Arbor viruses (19, 20, 5, 6, 7, 8, 9, 22, 23): (1) The release of hemagglutinin from infected tissues proves to be dependent on the pH and on the electrolytes of the extraction liquid; (2) The hemagglutinating activity is confined to a certain pH range, it is often dependent on the temperature and it mostly reveals a narrow erythrocyte spectrum. The enzymatic destruction of the cell receptors does not take place here. Normal sera are capable of inhibiting the hemagglutination through their content of nonspecific inhibitors; (3) The stability of the hemagglutinin is small in the pH zone of its optimum activity and is guaranteed only in case of alkaline reaction; (4) Experimental sustained passage of virus strains can cause a loss of the capability for hemagglutinin formation. Within this framework the hemagglutinins of various

Arboviruses do reveal characteristic differences, as was proved by Sabin and associates with respect to their optimum reactivity with erythrocytes and in accordance with the degree of their stability. On the basis of available but by no means complete investigations it would seem that the hemagglutinin of the yellow-fever virus would correspond most closely to that of the Japan-B-Encephalitis-Virus.

Summary

1. The hemagglutinin of the yellow-fever virus can be established quantitatively in infected human explants both in the tissue (through alkaline extraction with borate buffer) and in the liquid culture phase (after elimination of nonspecific inhibitors with freon).
2. Hemagglutinin can be established in the explanted tissue already before the cytopathogenic effect and it reaches its highest concentration at the time of the maximum infectiousness titer. The release of hemagglutinin into the culture medium takes place in a synchronized fashion with the progression of the cytopathogenic effect.
3. There is a certain proportionality between the hemagglutinin and the infectiousness titer.

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FIGURE APPENDIX

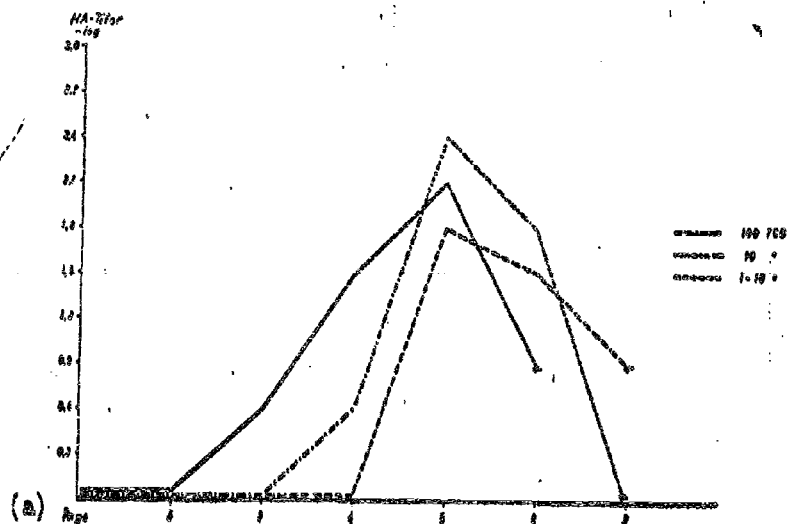


Figure 1. Explant: KB. Virus strain: Astib. N. 62nd passage. Legend: a-- days.

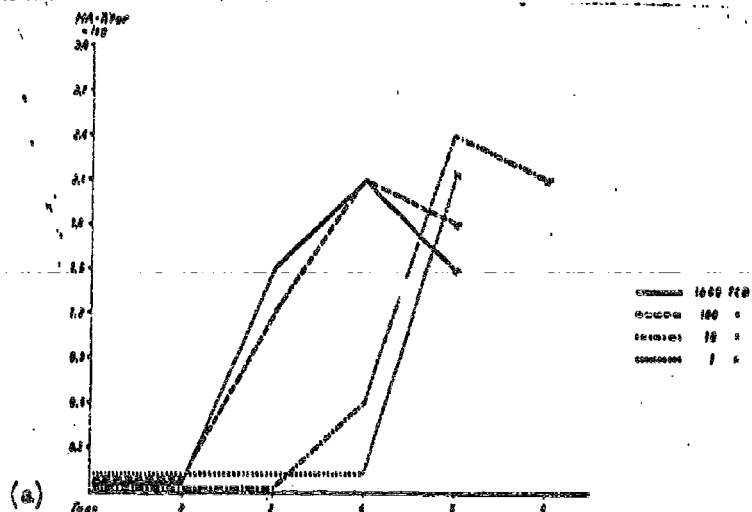


Figure 2. Explant: KB. Virus strain: Astib. N. 61st passage. Legend: a--days.

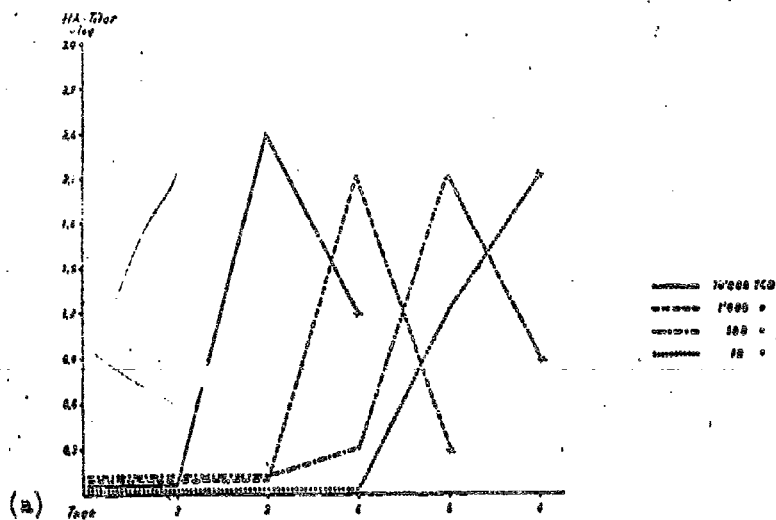


Figure 3. Explant: KB. Virus strain: Asibi II. 19th passage. Legend: a-- days.

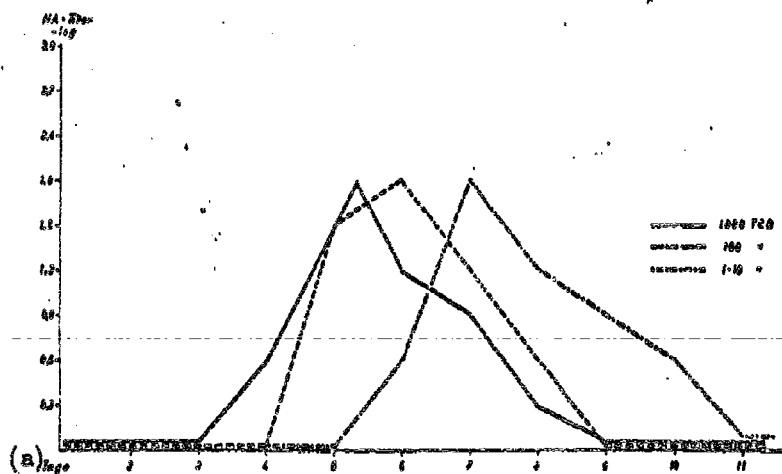


Figure 4. Explant: HeLa "Zürich." Virus strain: Asibi II. 20th - 2nd passage. Legend: a-- days.

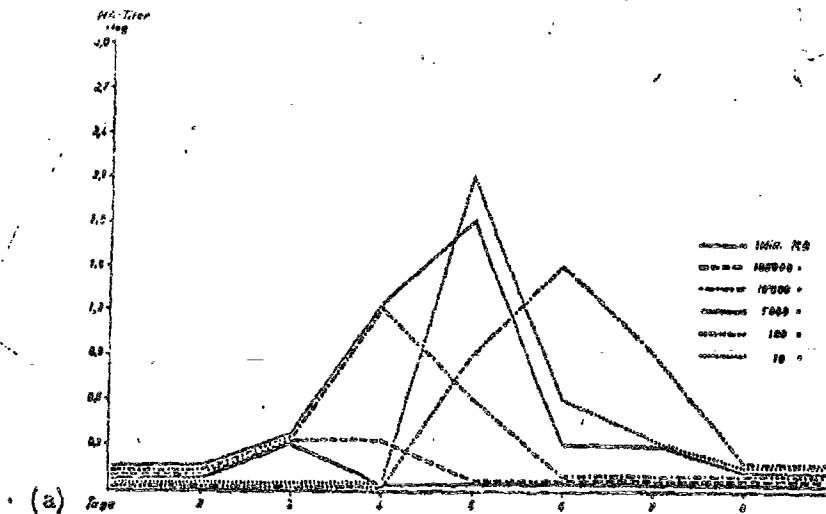


Figure 5. Explant: HeLa "Zürich." Virus strain: Asibi II, 20th - 1st passage. Legend: a-- days.

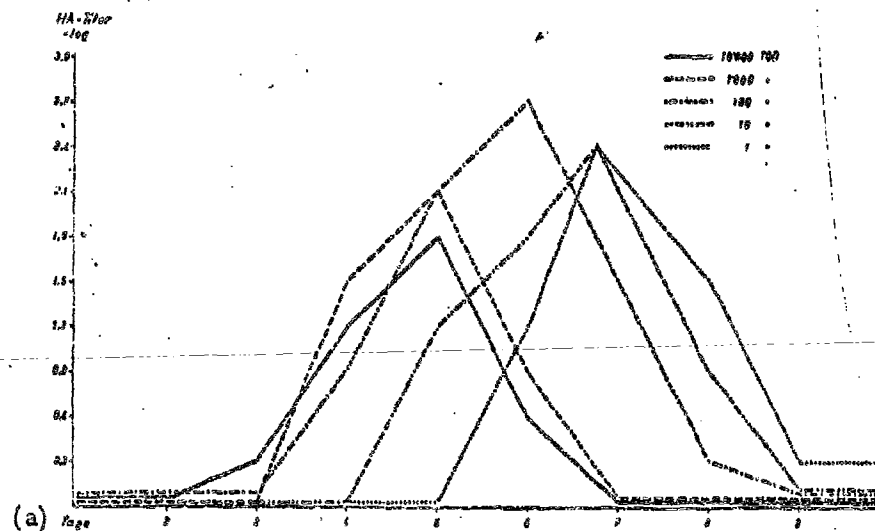


Figure 6. Explant: Amnion FL. Virus strain: Asibi II, 42th - 12th passage. Legend: a-- days.

Errata

1. Hallauer contribution: "Breeding and Modification of Atypical Poultry Test Virus (NDV) in Explants of Human Origin," Volume 8, No. 3, 1958, on page 399, first line: ID₅₀ log 0.0 - 2.0 (instead of 0.2).

2. Hallauer and Kronauer contribution: "Immunization Experiments With Experimentally Induced Variants of the Classical and Atypical Poultry Pest Virus," Volume X, No. 1, 1960, on page 47, Table 1.

	3rd Column	7th Column
Original virus (Allantois)	3.5 (instead of 5.3)	2.0 (instead of 3.8)